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The survival of silage inoculant lactic acid bacteria in rumen fluid

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ABSTRACT

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Aims: To determine whether lactic acid bacteria (LAB) used in inoculants for silage can survive in rumen fluid (RF), and to identify those that survive best.

Methods and Results: Twelve commercial silage inoculants were added at 10^7 CFU ml^{-1} to strained RF (SRF) taken from dairy cows, with and without 5 g l^{-1} glucose and incubated *in vitro* at 39°C. Changes in pH, LAB numbers and fermentation products were monitored for 72 h. In the inoculated RF with glucose, the pH decreased and numbers of LAB increased. The inoculants varied with regard to their effect on pH change and growth. In the SRF, both with and without glucose, the pH values of the inoculated samples were generally higher than those of the uninoculated controls throughout most of the incubation period. This may suggest a positive effect on the rumen environment.

Conclusions: LAB used in silage inoculants can survive in RF *in vitro*.

Significance and Impact of the Study: This is the first step in studying the probiotic potential of silage LAB inoculants for dairy cattle. The survival of these LAB in RF may enable them to interact with rumen microorganisms and to affect rumen functionality.

Keywords: lactic acid bacteria, silage inoculants, rumen fluid, probiotic effects.

INTRODUCTION

Ensiling is a preservation method for moist crops that is based on a natural lactic acid fermentation under anaerobic conditions, whereby epiphytic lactic acid bacteria (LAB) convert water-soluble carbohydrates into organic acids, mainly lactic acid. As a result, the pH decreases and the forage is preserved (McDonald *et al.* 1991).

Inoculants containing principally LAB are used as silage additives in order to improve preservation efficiency. Among the LAB, the most frequently used are homofermentative species such as *Lactobacillus plantarum, Enterococcus faecium* and *Pediococcus* spp. These are used because of their efficient utilization of the crop's water-soluble carbohydrates (WSC), intensive production of lactic acid and rapid decrease in pH (Weinberg and Muck 1996). Other LAB are also included, such as *L. buchneri*, a heterofermentative LAB which produces high concentrations of acetic acid in silage that inhibits fungi and thus preserves silages susceptible to spoilage upon exposure to air (e.g. Driehuis *et al.* 1999; Weinberg *et al.* 2002). An inoculation rate of 10^5–10^6 viable cells per gram crop is often sufficient for the inoculant LAB to overwhelm the epiphytic LAB and become the predominant population in the silage (Weinberg and Muck 1996).

In some cases, an effect of silage inoculation on animal performance has been observed. Feed intake, liveweight gain, feed efficiency and/or milk production were improved in 25–40% of the studies reviewed. The improvement of these parameters ranged from 5 to 11% (Muck 1993).

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The cause of improved animal performance is unclear. A considerable number of animal experiments using a single silage inoculant strain, *L. plantarum* MTD1 were performed in northern Ireland with grass silage (Keady and Steen 1994, 1995; Keady *et al.* 1994). The majority of these studies reported improved animal performance with silages inoculated with this strain, regardless of fermentation quality. When the inoculant was added to the silage immediately before feeding, there was no significant effect on dry matter (DM), nitrogen, neutral detergent fibre (NDF), or modified acid detergent fibre digestibility (Keady and Steen 1995). This might suggest that the benefits result from the silage fermentation rather than from effects of the LAB in the rumen itself. By contrast, in a recent study (Khuntia and Chaudhary 2002), dietary addition of a mixed culture of LAB increased DM intake, liveweight gain and DM digestibility in calves. Rumen pH was lower and lactic acid was higher following LAB feeding. Salawu *et al.* (2001) found that application of *L. plantarum* to pea–wheat silage increased the rate of nitrogen and NDF degradation in the rumen. Malik and Sharma (1998) inoculated rumen fluid (RF) with various microorganisms in the presence of wheat straw and concentrates, and showed that *L. acidophilus* improved DM and organic matter digestibility *in vitro* as compared with an untreated control.

The results of some of these studies suggest a possible probiotic effect of LAB used in inoculants for silage, the mechanism of which is yet unclear. One hypothesis is that specific LAB strains interact with rumen microorganisms to enhance rumen functionality and animal performance. Another hypothesis is that LAB which are used as inoculants for silage inhibit detrimental microorganisms in the silage. In this regard, it is well known that LAB produce a variety of antimicrobial substances such as bacteriocins (e.g. Vandenbergh 1993; Muller *et al.* 1996).

Unless the beneficial effects of silage-inoculant LAB were due to production of bacteriocins during ensiling, LAB ingested by the animals would have to survive in RF in order to affect rumen microflora. The purpose of the current study was to assess the survival of selected LAB from commercial silage inoculants in RF. This is the initial step in testing the hypothesis that LAB interact positively with certain rumen bacteria.

MATERIALS AND METHODS

Experimental

RF was collected for each experiment from two fistulated Holstein cows fed on total mixed ration containing 30% of DM alfalfa silage, 30% corn silage, 10% solvent soya bean meal, 30% ground shell corn and supplemental vitamins and minerals. The combined RF from the two cows was strained (SRF) through four layers of cheesecloth.

The SRF was subdivided into sterile Erlenmeyer flasks, each of which was inoculated with a commercial LAB silage inoculant at 10^7 CFU ml^{-1}. RF with no LAB inoculant served as a control. The inoculated RF was further subdivided and to one half was added sterile 50% (w/v) glucose solution to a final concentration of 5 g l^{-1}. The various treatments were added (7–9 ml) to sterile serum bottles which were flushed with CO_2 before sealing. The bottles were incubated at 39°C. At 6, 12, 24, 48 and 72 h after inoculation two bottles from each treatment were sampled for analysis.

Inoculants

The number of LAB cells in the dry products was determined before the experiments by suspending the inoculants in deionized water and pour plating serial dilutions into Rogosa SL agar or MRS (deMan Rogosa Sharpe) agar (Difco Becton Dickinson and Company, Sparks, MD, USA). The inoculants were applied by suspending an adequate weight (according to the LAB number in the product) in 100 ml of tap water and using 1 ml of the suspension to treat 200 ml of RF.

Table 1 lists the commercial inoculants used.

Analytical procedures

The enumeration of LAB was performed with pour plates. MRS agar was used for the *Enterococcus* spp.; all other inoculated treatments were enumerated on Rogosa SL agar. Plates were incubated in an anaerobic glove bag (0·80 N_2, 0·10 CO_2, 0·10 H_2 v/v) at 30°C for 3 days. Lactic acid and volatile fermentation products were determined by high-performance liquid chromatography (Muck and Dickerson 1988) on the 0, 48 and 72-h samples. Statistical analysis of data from a given time point was performed using the generalized linear model procedure of SAS (The SAS Institute, Cary, NC, USA) and where factors were significant, differences between treatments were determined by the least significant difference method ($P < 0·05$).

RESULTS

The inoculants were tested in two experiments: inoculants 1–5 in experiment 1, and 6–12 in experiment 2. The fresh SRF prior to inoculation contained LAB counts of 6·2 and 5·7 (5·9 on MRS agar) $\log_{10}$ CFU ml^{-1} and pH values of 5·70 and 5·57 for experiments 1 and 2, respectively. Thus, inoculation provided approximately a 10-fold increase in LAB numbers over background levels in the control treatment.

Table 1 Inoculants used in the two experiments

Number	Inoculant	Source	Abbreviation
1	Biomax5™ containing *Lactobacillus plantarum*	Chr. Hansen Biosysystems (Milwaukee, WI, USA)	LPC
2	Pioneer 1174™ containing *Lactobacillus plantarum* and *Enterococcus faecium*	Pioneer Hi-Bred International Inc. (Des Moines, IA, USA)	LP/EF
3	Pioneer 11A44™ containing *Lactobacillus buchneri*	Pioneer Hi-Bred International Inc.	LBP
4	*Lactobacillus buchneri*	Biotal Canada Limited (Calgary, AB, Canada)	LBB
5	Biomate LP/PC™ containing *Lactobacillus plantarum* and *Pediococcus cerevisiae*	Chr. Hansen Biosysystems	LP/PC
6	*Lactobacillus plantarum* MTD1	Ecosyl (Yorkshire, UK)	LPE
7	*Pediococcus pentosaceus*	Ecosyl	PPE
8	*Enterococcus faecium* (Q)	Agri-king (Fulton, IL, USA)	EFQ
9	*Enterococcus faecium* (C)	Agri-king	EFC
10	*Pediococcus pentosaceus*	Agri-king	PPA
11	*Lactobacillus pentosus*	Agri-king	LPeA
12	*Lactobacillus plantarum*	Agri-king	LPA

Table 2 pH and LAB ($\log_{10}$ CFU ml^{-1}) in rumen fluid after incubation with silage LAB for 72 h

Experiment	Glucose (g l^{-1})	Final pH	Final LAB	Lowest final pH	Highest final pH	Highest LAB	Lowest LAB
1	0	5·39–5·47	6·3–6·8	LPC	LBP*	LP/PC	LBB
	5	5·18–5·25	7·1–7·3	LPC and LBB	LBP	LP/PC	LBP
2	0	5·41–5·50	6·0–7·4	LPE and PPE	EFC	C and LPE	PPA and LPeA*
	5	5·14–5·27	6·6–7·4	EFC and PPA	PPE	EFC and LPA	LPE and PPE*

C, control.
*Differences in final pH or LAB between inoculated treatments within a row were statistically significant ($P < 0·05$).

The final pH and LAB numbers are summarized in Table 2. In the SRF without glucose supplementation final pH was numerically lower than the pH values of the fresh RF by 0·07–0·31 units. With glucose addition, there was a decrease in pH compared with the pH values of the fresh RF, ranging from 0·30 to 0·52 units. Final pH was affected significantly ($P < 0·05$) by glucose addition in all experiments and by inoculant in experiments 1.

Final LAB counts were within ±1·0 log units of initial values with the exception of the control without glucose in experiment 2 where counts rose approximately to 1·5 log units. Nevertheless, glucose addition significantly raised LAB counts, but inoculant did not affect final LAB counts in either experiment. The interaction of glucose and inoculant was significant in experiment 2, and significant differences between the LAB counts from different inoculants with and without glucose were observed (Table 2).

The inoculants resulting in the lowest pH values tended also to have the highest LAB numbers; and those resulting in the highest pH values usually also had the lowest LAB counts, regardless of glucose addition.

Figures 1 a and 2 a show the change in pH during incubation of SRF (experiments 1 and 2, respectively). In both experiments, glucose supplementation resulted in lower pH values throughout the incubation period, as compared with no glucose addition. A striking observation is that the inoculants resulted in higher pH values than the respective controls throughout the incubation period. Without glucose, inoculants LBB, and LP/PC provided similar buffering to inoculant LPC, and inoculants and LP/EF and LBP were similar. Without glucose in experiment 2, most of the inoculants performed like inoculant EFQ; inoculant PPE had an intermediate level of buffering compared with LPE and EFQ. With glucose, pH time courses for all other inoculants in experiment 1 were similar to that of inoculant LPC (Fig. 1a), and all inoculants in experiment 2 had similar pH time courses (Fig. 2a).

Figures 1b and 2b give the change in LAB with time in the SRF at 39°C. In the inoculated treatments without

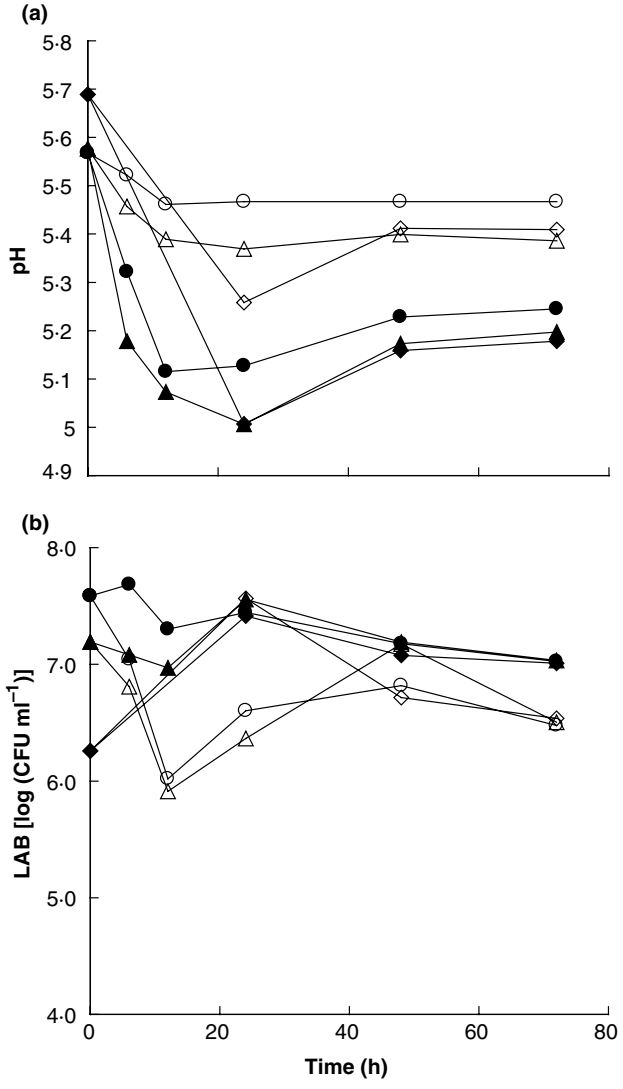

Fig. 1 Changes in (a) pH and (b) LAB counts in strained rumen fluid during incubation with inoculants in experiment 1. ◆, Control; ▲, LPC; ●, LBP. Open symbols, without glucose; solid symbols, with glucose

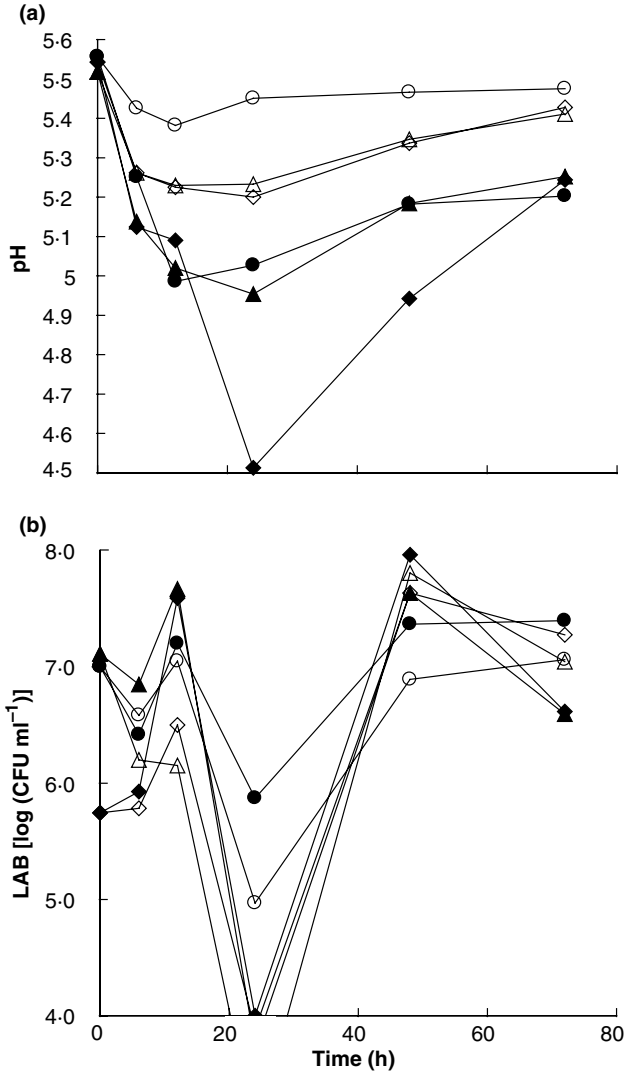

Fig. 2 Changes in (a) pH and (b) LAB counts in strained rumen fluid during incubation with inoculants in experiment 2. ◆, Control; ▲, LPE; ●, EFQ. Open symbols, without glucose; solid symbols, with glucose. The lowest dilution plated was the 10^4 dilution so that points below 4·0 $\log_{10}$ CFU ml^{-1} were below detectable level

glucose addition, there was generally a decrease in LAB counts relative to the inoculation rate (10^7 CFU ml^{-1}) especially in the initial 12–24 h, followed by a recovery at 48 h to near initial values. In experiment 2 at 24 h, the LAB numbers decreased below the detectable levels (10^4 CFU ml^{-1}) in many treatments. This, however, appeared to be an underestimate of true LAB because the plates contained many pinpoint colonies that did not develop into normal colonies with extended incubation. With glucose, the LAB counts in inoculated treatments declined less in the first 12 h and generally were higher at a given time point than those in corresponding vials without glucose. In experiment 1, LAB numbers in the control samples increased from 6·2 $\log_{10}$ CFU ml^{-1} to 7·6 and 7·4, without and with glucose, respectively, after 24 h and decreased thereafter. In experiment 2, the LAB numbers of the control increased both with and without glucose over the first 48 h (ignoring the anomalous 24-h points) before declining over the last 24 h of the experiment.

The fresh SRF contained 97–98 mM acetate, 35–38 mM propionate and 22–24 mM butyrate. These concentrations are typical of those in the rumen (Van Soest 1994). The concentrations of the volatile fatty acids (VFA) in the control samples increased during incubation and reached 110–148 mM acetate, 43–54 mM propionate and 32–49 mM butyrate after 72 h. The levels in the inoculated SRF after

Table 3 Average fermentation products in strained rumen fluid after 72 h incubation

Treatment	Total VFA (mM)	Molar fraction of total VFA		
		Acetate	Propionate	Butyrate
Experiment 1				
Control	191	0·587	$0·236^{A}$	$0·176^{C}$
LPC	193	0·594	$0·227^{B}$	$0·179^{BC}$
LP/EF	190	0·589	$0·225^{B}$	$0·186^{AB}$
LBP	195	0·596	$0·230^{AB}$	$0·175^{C}$
LBB	196	0·590	$0·223^{B}$	$0·188^{A}$
LP/PC	196	0·588	$0·223^{B}$	$0·189^{A}$
Without glucose	191	0·611*	0·222	0·167
With glucose	195	0·570	0·233*	0·197*
Experiment 2				
Control	246^{A}	0·598	$0·207^{C}$	0·195
LPE	226^{B}	0·569	$0·222^{BC}$	0·210
PPE	196^{CD}	0·576	$0·224^{BC}$	0·199
EFQ	190^{DE}	0·587	$0·221^{BC}$	0·191
EFC	186^{E}	0·586	$0·225^{B}$	0·189
PPA	191^{DE}	0·587	$0·222^{BC}$	0·191
LPeA	193^{D}	0·574	$0·232^{AB}$	0·194
LPA	200^{C}	0·568	$0·246^{A}$	0·185
Without glucose	200	0·606*	0·224	0·170
With glucose	206*	0·555	0·226	0·218*

Total VFA is the sum of acetate, propionate and butyrate.
Different letters within a column and experiment indicate statistically significant differences ($P < 0·05$) among inoculant treatments.
*Indicates statistically significant effects ($P < 0·05$) because of glucose addition.

incubation were 105–139 mM acetate, 40–52 mM propionate and 28–52 mM butyrate. No lactate was found in any of the samples of the SRF. Low levels of ethanol (1·0–1·4 mM) were detected in the inoculated glucose-added samples of experiment 1 with the exception of inoculant LP/EF.

Table 3 shows the average VFA concentrations at 72 h. Statistically significant effects were observed for glucose addition and/or inoculant. The only significant inoculant–glucose interaction terms were for butyrate in experiment 1 and total VFA in experiment 2. In experiment 1, propionate and butyrate was significantly affected by inoculation, decreasing and increasing, respectively, relative to the controls. In experiment 2, in which VFA levels in the control samples were high, inoculation suppressed VFA formation. Inoculation in experiment 2 increased the molar fraction of propionate, and there were consistent trends for reduced acetate resulting from inoculation. In both experiments, glucose addition reduced acetate formation and increased propionate and butyrate formation. The significant interaction effect with butyrate in experiment 1 was due to the controls producing a smaller increase due to glucose addition, relative to the increases observed with the inoculated samples. The significant interaction for total VFA in experiment 2 was due to the some inoculants (EFC, PPA, LPeA and LPA) producing larger increases in VFA from glucose addition than the other treatments.

DISCUSSION

The current experiments were conducted as part of a broader research objective, which is to find out how LAB silage inoculants enhance ruminant performance. The first step in this context is to determine whether LAB included in silage inoculants can survive and grow under rumen-like conditions. The SRF is a model that simulates conditions prevailing in the rumen, as it allows competition and interactions between inoculant LAB and the indigenous rumen microflora under environmental conditions (temperature, pH, concentrations of VFA and other chemicals) similar to those in the rumen. The inoculation rate of the SRF (10^{7} ml^{-1}) was set to be comparable with LAB numbers ingested by cows who receive 45 kg silage (wet weight) daily in their rations (assuming that silage contains 10^{6}–10^{7} LAB g^{-1}, and rumen volume is 150 l). LAB are typically not important in a forage-fed rumen, except when large amounts of soluble carbohydrates or starch are available (Van Soest 1994). However, results of this study indicate that the tested LAB were able to survive and in many cases grow in the SRF. As expected, glucose addition markedly enhanced the survival of the inoculant LAB in the RF, suggesting that silage-inoculant LAB strains can compete effectively with ruminal microflora in the presence of exogenous glucose. Some inoculant LAB strains (e.g. inoculants LP/PC, LPE and EFC) grew better than others. In the current study, inoculant LPE (*L. plantarum* MTD1) also resulted in the highest concentrations of VFA in the SRF relative to other inoculants. Interestingly, this strain has been found to impart beneficial effects on cattle performance in studies performed in Northern Ireland (Keady *et al.* 1994; Keady and Steen 1995).

The fact that no lactic acid was found in the inoculated SRF does not necessarily suggest that the inoculants did not produce lactic acid in that environment. It could well be that lactate produced by the inoculants was immediately converted by rumen microorganisms to other metabolites (VFA). Lactate levels in the rumen are usually lower than those of VFA (Van Soest 1994). There were also shifts in fermentation in both experiments relative to inoculation. Relative to controls, inoculated treatments had elevated butyrate levels in experiment 1 and higher propionate levels in experiment 2. Both are potential end-products of lactate fermentation depending on the dominant ruminal microbial strain. Alternatively, the inoculant LAB may not have fermented the glucose, but might have affected the microbial population which did.

The observation that the pH of the LAB inoculated samples was consistently higher than the respective controls was surprising because in silage, LAB cause a rapid decrease in pH as a result of their production of organic acids, mainly lactic acid. Strains also varied in their ability to buffer pH, both with and without supplemental glucose. Inoculants LBP, LP/EF and EFQ were best at maintaining pH near to their initial values. This phenomenon suggests that the mode of action of the inoculants in the RF is more likely in influencing which rumen microorganisms predominate rather than by direct fermentation of substrates by LAB in the rumen. Higher rumen pH might certainly enhance the functionality of specific rumen microorganisms, especially in cases when the pH decreases following high-energy feeding (Van Soest 1994). In addition, improved fibre digestibility of inoculated silage in cattle has been reported (Muck 1993), and this buffering effect may be a possible explanation, as growth of ruminal fibrolytic bacteria is known to be inhibited at pH<6 (Weimer 1996).

There might be a difference in pH values of fresh RF which could evolve from seasonal variations, feeding or individual cow-to-cow variations. How these variations might affect the LAB mode of action in the rumen is not as yet clear and warrants more research. Our hypothesis is that lower RF pH values would favour LAB in their competition with rumen microorganisms. In order to elucidate the mechanism by which LAB impart beneficial probiotic effects on ruminants, more research is needed which would study their effect on fibre degradation and possible bacteriocin production.

CONCLUSIONS

The results of the current study indicate that silage-inoculant LAB can survive in RF. They bring about some changes in the RF such as changes in pH and VFA composition. How these changes affect animal performance is not as yet clear and needs more research.

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